University of Zurich[UZH]

Zurich Open Repository and Archive
University of Zurich
University Library
Strickhofstrasse 39
CH-8057 Zurich
www.zora.uzh.ch

Year: 2016

Determination of single cell lag times of Cronobacter spp. strains exposed to different stress conditions: impact on detection

Margot, Heike ; Zwietering, M H ; Joosten, Han ; Stephan, Roger

Abstract: The variability of stress resistance and lag time of single cells can have a big impact on their growth and therefore on the probability of their detection in food. In this study, six strains of Cronobacter spp. were subjected to heat, acid and desiccation stress and single cell lag times were determined using optical density measurements. The duration of lag time was highest after acid stress and did not correlate to stress resistance. The effect that the inactivation caused by stress and an extended lag time had on the projected cfu level reached after enrichment was simulated in different scenarios. For most strains, an enrichment time of 18h was sufficient for stressed cells to reach the suggested minimum level of cell inoculum for the Cronobacter screening broth detection. Particular strains may require longer recovery periods. Further, probability calculations showed that the number of samples taken from a batch may have an important effect on detection probability, especially at low contamination rates. Therefore, in addition to increasing the recovery period, increasing the number of samples is a suitable strategy to improve detection.

DOI: https://doi.org/10.1016/j.ijfoodmicro.2016.08.002

Posted at the Zurich Open Repository and Archive, University of Zurich
ZORA URL: https://doi.org/10.5167/uzh-129948
Journal Article
Accepted Version

Originally published at:
Margot, Heike; Zwietering, M H; Joosten, Han; Stephan, Roger (2016). Determination of single cell lag times of Cronobacter spp. strains exposed to different stress conditions: impact on detection. International Journal of Food Microbiology, 236:161-166.
DOI: https://doi.org/10.1016/j.ijfoodmicro.2016.08.002

Determination of single cell lag times of *Cronobacter* spp. strains exposed to different stress conditions: impact on detection

H. Margot[1], M. H. Zwietering[2], H. Joosten[3,4], R. Stephan[1]

[1]Institute for Food Safety and Hygiene, Vetsuisse Faculty University of Zurich, CH-8057 Zurich, Switzerland

[2]Laboratory of Food Microbiology, Wageningen University, 6700 AA Wageningen, the Netherlands

[3]European Chair in Food Safety Microbiology, Wageningen University, 6700 AA Wageningen, the Netherlands

[4]Imdea Food Institute, E-28049 Madrid, Spain

* Corresponding author:

R. Stephan, Institute for Food Safety and Hygiene, Vetsuisse Faculty University of Zurich, Winterthurerstr. 272, CH-8057 Zurich, Switzerland, Phone 0041-44-6358651, fax 0041-44-6358908, e-mail stephanr@fsafety.uzh.ch

Abstract

The variability of stress resistance and lag time of single cells can have a big impact on the growth and therefore on the probability of detecting bacteria in food. In this study, six strains of *Cronobacter* spp. were subjected to heat (10 min at 55ºC), acid (2 h at pH 4) and desiccation stress (2 days in sterile $CaCO_3$) and single cell lag times were determined using optical density measurements. Longest lag times were found after acid treatment (12.8 h for strain E288). However, the effect on bacterial inactivation of the different stresses applied and the change in lag times for each strain did not correlate for heat and acid stress. There was a negative correlation for desiccation stress.

By simulating different scenarios of one cell in a sample experiencing inactivation and increased lag time we showed that the detection of *Cronobacter* might fail because the detection limit of the second selective enrichment in Cronobacter screening broth might not be reached. In general, such simulations can help to visualize the effects of inactivation and increased lag for a number of strains and thereby compare behaviour of these strains.

Keywords enrichment, sampling plan, strain variability, bacterial inactivation, powdered infant formula

1. Introduction

Various studies showed that bacterial cells react to different stresses with an extended lag phase due to repair time (Smelt et al., 2002; Stephens et al., 1997). Dupont and Augustin (2009) investigated the influence of diverse stress conditions common in the food industry on the single cell growth probability and lag time distribution of *Listeria monocytogenes*. They found an increase in mean and variability of lag times in injured cells. During lag phase bacteria adapt to the new environmental conditions. This process includes the repair of macromolecular damage accumulated during stationary phase (or other stresses) and the synthesis of cellular components that are necessary for growth (Dukan and Nystrom, 1998).

The mean-value of single-cell lag times is not automatically equal to the population lag time and the lag time of the population does not necessarily give information on the variability of the single-cell lag time in a population (D`Arrigo et al., 2006) Therefore, knowledge about single cell lag time is critical in view of the detection probability in products with very low contamination levels. Experiments with single cells have given the opportunity to investigate the environmental effects of the distribution of lag times of single cells (Pin and Baranyi, 2008). One way to inoculate every well with approximately one cell is by serial dilution. With time-to-detection information obtained from OD measurements, lag time can be calculated if the cell concentration at the detection level and specific growth rate is accurately known (Dupont and Augustin, 2009; Guillier et al., 2005).

There are studies describing the influence of stress factors on single cells of *Listeria monocytogenes*, *Lactobacillus* and *E. coli* (Gnanou Besse et al., 2000; Li et al., 2006; Smelt et al., 2002). However, studies analysing the influence of stress on different species and strains of *Cronobacter* are scarce and often obtained from predictive modelling (Miled Bennour et al., 2011; Xu et al., 2015). In this study we describe the influence of stress factors relevant to powdered infant formula (PIF) production and reconstitution on bacterial inactivation and lag

time of *Cronobacter* spp. single cells from different species, aiming to show the impact on the probability of detection in different scenarios.

2. Materials and Methods

2.1. Strains

Six strains of different *Cronobacter* species were chosen for the experiments (Table 1). The strains were chosen to represent the variability of different species of *Cronobacter*. Working cultures were made from frozen stocks (BHI (Oxoid CM1135, Basingstoke, United Kingdom) with 20% glycerol) and maintained on blood agar plates (Difco Columbia blood agar base, 5% sheep blood, Oxoid) at 4± 1°C.

2.2. Stress application

Heat stress: 1 ml of an overnight culture grown in brain heart infusion broth (BHI, Oxoid), with a concentration of ca. 10^9 cfu/ml, was added to 9 ml buffered peptone water preheated to 55ºC in a water bath and incubated for 10 min. Afterwards, the culture was diluted to single cell level in cold BPW to stop the heat exposure and added to the wells of a 96 well plate. This stressed culture was also diluted in 0.9% saline and plated on TSA to determine cfu counts. Cell counts of each overnight culture were determined by plate counting on TSA.

Acid stress: 1.5 ml of an overnight culture grown in BHI was centrifuged at 13,000 x g for 10 min. The supernatant was discarded and the pellet was resuspended in 1.5 ml LB broth (Oxoid) adapted to pH 4.0 with lactic acid. The suspension was incubated at room temperature for 2 h and diluted to single cell level in BPW (pH=7) and added to the wells of a 96 well plate. The stressed culture was also diluted in 0.9% saline and plated on TSA to determine cfu counts.

Desiccation stress: Desiccation stress was applied as described previously (Margot et al., 2015). Briefly, 30 ml of a BHI overnight culture were centrifuged for at 3,200 x g for 12 min. The supernatant was discarded and the pellet mixed with small amounts of sterile $CaCO_3$ until a final amount of 15g was achieved. The powder was stored in a desiccator with silica gel for two days before the experiment. The stressed culture was diluted in 0.9% saline and plated on TSA to determined cfu counts before and after two days of storage in the desiccator. The powder was also diluted in BPW to reach the desired inoculum in the 96 well plate.

2.3. Population growth rate

The population growth rates were determined as follows. Serial dilutions were performed in BPW in order to obtain 10^2 -10^3 cfu/ml. The samples were incubated with agitation for 24 h at 37ºC. 100 µl samples were removed periodically for 24 h and spread plated on TSA plates. The plates were incubated at 37ºC for 24 h. The growth rate was calculated using ComBase DMfit software (United States Department of Agriculture). Since bacterial counts were in log10 cfu/g, the maximum growth rate as determined by DMfit was in log10 cfu/ml/h. Therefore the values were converted to maximum specific growth rate (1/h) by multiplying by ln (10). The experiment was performed in three independent experiments.

2.4. Optical density calibration curve

In order to determine N_d, the cell concentration corresponding to an OD of 0.50, a diluted overnight culture of each strain was incubated in the BioTek (Bio Tek, Luzern, Switzerland) instrument with agitation and the OD was recorded at 37ºC and OD_{600nm} every 10 min. 100 µl samples were taken just after each OD measurement and plated on TSA. The cell concentration corresponding to an OD of 0.50 was N_{E151} = 8.0 log cfu/ml, N_{E288} = 7.4 log

cfu/ml, N_{E465} = 7.9 log cfu/ml, N_{E681} = 7.5 log cfu/ml, N_{E688} = 7.5 log cfu/ml N_{E776} = 7.7 log cfu/ml.

2.5. Determination of single cell lag times

Cultures of stressed cells were diluted in BPW in order to reach a maximum concentration of 2.1 cfu/ml in the final dilution. Cells without application of stress were revived from frozen stocks, cultured in BPW for 24 h at 37ºC and diluted in BPW to the same level. The wells of a 96-well plate were inoculated with 200 µl of this suspension to obtain a target value of 0.42 cfu/well. Assuming a Poisson distribution of the cells in the wells, a maximum concentration of 0.42 cell/well should correspond to 34% of the wells showing growth (Francois et al., 2003). By estimation, less than 20% of the wells showing growth should contain more than 1 cell. 96-well plates were incubated in the BioTek reader for 24 h at 37ºC with shaking. OD at 600nm was measured every 10 min. A minimum of three independent replications per experimental set-up was performed to obtain approx. 80 lag time values total per strain and stress factor. The time to detection in each well (time to reach an OD of 0.50) was calculated for each experiment with Microsoft Excel. Individual cell lag times were estimated from time to detection data according to protocols by Dupont and Augustin (2009) and Guillier et al. (2005). Assuming an exponential bacterial growth at a constant specific growth rate (µ) until the detection time, T_d is related to the lag time of the culture with N_0 cells (in this case 1 cell in 200 µl) (lag) by the following formula proposed by Baranyi and Pin (1999):

$$lag = T_d - (\ln(N_d) - \ln(N_0)) / \mu$$

The distribution of the individual lag times should be reflected by the distribution of the detection times when N_0=1 (Metris et al., 2003).

2.6. Simulating the impact of sublethal stress on the lag time and growth of single cells

The growth of a single *Cronobacter* cell in an enrichment of 100 ml was simulated taking into account the population growth rate and the bacterial inactivation caused by stress, which was determined for each strain. Both the minimum, maximum and mean individual lag time determined in this study was included in the simulation to visualize the effect on the final cell counts. The probability of detection of six *Cronobacter* strains was calculated taking into account the final cell counts. The growth limit was set at 10^9 cfu/ml because this is the cell count at which stationary phase is usually reached.

3. Results and Discussion

3.1 Population growth rates

The following specific growth rates were determined at 37ºC in BPW:

E151: 1.94± 0.09 h^{-1}; E288: 1.88± 0.29 h^{-1}; E465: 2.21± 0.07 h^{-1}, E681: 1.89± 0.23 h^{-1}; E688: 1.89± 0.09 h^{-1}; E776: 1.83± 0.44 h^{-1}. In literature, growth rates of *Cronobacter* spp. determined at 37ºC range from 1.80 h^{-1} to 2.72 h^{-1} in BHI (Miled Bennour et al., 2011) and from 1.32 h^{-1} to 2.89 h^{-1} in PIF (Kandhai et al., 2006). The great variation can be caused by various factors as e.g. different growth media, incubation temperatures and species/strain used (Swinnen et al., 2004). In this study, growth rates were determined in BPW, which is a medium less rich than BHI and reconstituted infant formula. Still, growth rate data are within ranges as given in literature. BPW was chosen because it is prescribed in the ISO method for the non-selective enrichment of *Cronobacter* spp. Differences in growth rates between studies can also be explained by the method that was applied to determine the growth rate. Miled Bennour et al. (2010) used OD measurements for this purpose and obtained values of 1.72 to 2.34 h^{-1}, whereas the values mentioned above were determined by plate counts. The correct estimation of the growth rate has a significant effect on the determination of lag time (Aguirre et al., 2014).

3.2 Distributions of single cell lag time

Distributions of single cell lag times for each strain after exposure to desiccation, acid and heat stress are shown in Figure 1. For the different strains, distribution of lag times varied greatly. Whereas for strains E151, E465, E681 and E688, lag times are close, values obtained with E288 and E776 are clearly separated. Lag times after heat stress seemed to be less scattered for strains E151, E288 and E465. Other authors described the effect of stress factors like pH and temperature on single cell lag time and its distribution (Francois et al., 2003; Metris et al., 2003; Smelt et al., 2002). The common finding was that increasing intensity of stress treatment lengthened lag time and increased lag time variability. Using OD measurements to determine bacterial lag times is not always considered to be ideal because variation in bacterial cell size and inaccurate biomass detection can result in misleading data (Rolfe et al., 2012). In addition, turbidity measurements have very high detection limits, which limit the range in which data are accurately obtained. However, single cell lag time cannot be deduced from population lag and large quantities of replicate measurements are needed. Due to these reasons, viable counting cannot be used. Another method circumventing the above-mentioned limitations was described by Elfwing et al. (2004). With the use of a flow chamber in which the cells are attached to a solid surface the divisions of single cells and the distribution of lag times can be monitored.

3.3 Correlation of lag times and inactivation

Both single cell lag time and log inactivation by stress are shown in Figure 2. Mean lag times per strain after acid stress were between 2.7 and 9.5 h. Mean inactivation values were between 1.9 and 4.0 log. When treated with elevated temperature, mean lag times were between 0.8 and 4.3 h. Mean inactivation was between 3.0 and 4.1 log cfu/ml. Desiccation stressed cells

showed mean lag times reaching from 0.6 to 4.5 h. Mean inactivation values were between 2.3 and 4.2 log.. Mean lag time for all strains was 5.0 h after acid stress, 2.5 h after heat stress and 2.7 h after desiccation stress. Mean log inactivation was 2.9 log after acid stress, 3.5 log after heat stress and 3.3 log after desiccation stress. Conclusively, acid stress did cause the longest lag times when at the same time inactivation was similar for all stress factors. The data suggest that lag times of cells are not correlated to inactivation for heat and acid stress (Pearson`s correlation coefficient of 0.09 and -0.25, respectively). There was a very strong negative correlation for desiccation stress (Pearson`s correlation coefficient of -0.87). In addition, we showed that some strains can be more resistant to a certain stress factor and on the other hand have pronounced sensitivity for a different stress. Guillier et al. (2005) measured lag times of *Listeria monocytogenes* after treatment with 9 stresses that all caused an inactivation of 1.5 log cfu/ml. Even though inactivation was identical, length of lag varied significantly (2.1 h – 16.1 h). Inactivation of *Cronobacter* spp. by drying was determined by Dancer et al. (2009). They found that cells dried in TSB experienced an inactivation between 0.8 and 1.5 log after 3 days, values were lower when cells were desiccated in infant formula (0.5-1.3 log). Caubilla-Barron et al. (2004) studied the desiccation resistance of *Cronobacter* spp. in freeze- and air-dried infant formula and found a 2.0 log decrease. Gurtler and Beuchat (2005) investigated the heat resistance of four *Cronobacter* spp. strains and found inactivation of 0.4 to 1.9 log cfu/ml after 5 min at 55ºC. These values are close to the ones obtained in this work when the values are extrapolated to 10 min heat treatment (0.8-3.8 for Gurtler and Beuchat (2005) and 3.0-4.1 for our results). Xu et al. (2015) found population lag times of *Cronobacter turicensis* of 6.2 h of heat treated cells incubated at 22ºC. Individual lag times of *Cronobacter* at 37ºC had a mean of 4.5 h for 12 fitted shape parameters and population lag times between 2.25 and 3.71 h. Kandhai et al. (2006) estimated population lag times of 1.7± 0.4 h of *Cronobacter* spp. in reconstituted infant formula at 37ºC. *Cronobacter* spp. has the ability to survive under dry conditions when compared to other *Enterobacteriaceae*. The

desiccation tolerance of *Cronobacter* spp. increases its competitive advantage in production facilities and is an explanation for the contamination of infant formula (Breeuwer et al., 2003). It was shown that the thermal tolerance varies between strains, which is confirmed by our data (Nazarowec-White and Farber, 1997). The disparity between the different *Cronobacter* species and strains and the resulting variance in stress resistance and lag time presents a great challenge. Therefore, the collection of data, covering a wide selection of strains, should be the main focus of research activity.

3.4 Simulating the impact of sublethal stress on the lag time and growth of single cells and their detection

In order to visualize the effect of an increased lag time due to stress encountered during food production, different scenarios with desiccated cells were simulated (Figure 3). Desiccation stress was chosen, as it is the most relevant stress on *Cronobacter* along the powdered infant formula production line. The inactivation has an impact on the probability of the presence of a viable cell in the sample. Simulations take into account the maximum specific growth rate of each strain and mean individual lag time. The simulation is based on the assumed presence of 1 cell in a sample of 10 g diluted in 100 ml enrichment medium.

This simulation shows that after an incubation of 18 h, cell counts will for some *Cronobacter* strains remain at a very low level if the maximum lag time is assumed. Cell counts of strain E288 will remain below 4 log cfu/ml when the maximum lag time is anticipated. When the "worst case" scenario was simulated, a single cell of *Cronobacter* E151 with a maximum lag of 9.7 h (as determined) would have grown to 4.99 log cfu/ml after a 18 h incubation. In the standard detection method for the detection of *Cronobacter* spp. from infant formula, 10g of powder are enriched in BPW for 18± 2 h at 37ºC. Afterwards, 0.1 ml is transferred into the selective broth CSB (Cronobacter screening broth). According to Iversen et al. (2008), 10^4 cfu/ml in the enrichment are required for the slowest growing strain to change the colour of

CSB, which is based on the fermentation of sucrose. The time to reach this level starting from a concentration of 0.01 cfu/ml (6 log cfu/ml increase) is shown in Table 2. According to these values, the enrichment time of 18± 2 h is sufficient to reach the required concentration if the mean lag time is assumed. For strain E288 this is not the case if the longest lag time is occurring. Therefore, an extended recovery period will not have a great effect on detection in most cases, but in specific situations it might result in a false negative.

The probability of detecting a positive sample was calculated assuming different contamination rates and sampling plans (Figure 4). The fraction of positive samples of 0.11 for a 10g sample size was used for the calculation as determined by Jongenburger et al. (2011), who sampled a contaminated batch of powdered infant formula. The influence of increasing the number of samples on the probability of detection was simulated in dependency of the contamination rate. The scenarios show that with an increased number of samples the probability to detect a positive sample with a contamination rate of 0.11 is increased from 0.69 to 0.997. On the other hand it is shown that at very low contamination levels, 250 samples would be necessary to achieve a comparable probability of detection (P= 0.92).

Miled Bennour et al. (2011) estimated the probability of detecting *Cronobacter* with CSB with either 1 or 3 cells in a 10g sample. In case a 10g sample was contaminated with only 1 cell, the probability of detection would be 0.212± 0.019 according to them. This means that of 100 samples of 10g, each contaminated with 1 cell of *Cronobacter*, only 20 would be detected. The simulation of different scenarios can visualize the effect of an extended lag time if the contamination level is very low. The literature shows that levels of *Cronobacter* spp. found in powdered infant formula were between -3.84± 0.696 log cfu/g (Jongenburger et al., 2011). Therefore, our simulation shows a realistic situation for the detection of *Cronobacter* spp. from powdered formula. Growth rates determined in pure BPW might be lower than in the actual sample containing infant formula, where more nutrients are vitamins available to

the bacteria. However, growth rates determined in BPW did not differ considerably from literature data obtained in diluted PIF. Even though bacterial counts in PIF are usually very low, it is not a sterile product. The background flora growing during the enrichment, can reduce the outgrowth of *Cronobacter* before the end of incubation due to depletion of nutrients or accumulation of toxic compounds in the medium. This was shown by Miled Bennour et al. (2011), who simulated the growth of *Cronobacter* in a 10g and 100g sample in the presence of the background flora.

4 Conclusion

The variability of lag time of individual cells following exposure to stress can have an impact on the growth and detection of bacteria. This is even more important in conditions of very low contamination levels of a product (Guillier and Augustin, 2006) since stressed single cells might be missed during detection due to an increased lag time.

The aim of this study was to show the influence of inactivation by stress and an increased lag time on the detection probability of single *Cronobacter* cells.

The presented data demonstrate that the *Cronobacter* strains chosen for this study displayed great variation in lag time and stress resistance. The duration of lag time was highest after acid stress and did not correlate to stress resistance for heat and acid stress. By considering different scenarios of one cell in a sample experiencing inactivation due to desiccation and increased lag time, we showed that the detection of *Cronobacter* might fail because the detection limit of the second selective enrichment might not be reached. However, this was only the case for one strain. If the incubation time would be set at 21 h, also for this strain even at the highest found individual lag time a sufficient level would be reached. It was shown that the number of samples taken did have a very large effect on detection probability,

especially at low contamination rates. Therefore increasing the number of samples is also a suitable strategy to improve detection.

In general, such simulations can help to visualize the effects of inactivation and increased lag for a number of strains and thereby compare their behaviour.

In future studies, data considering also growth experiments with the product should be collected to respect also the influence of the product and of the background flora.

Acknowledgements: We thank Paul Torgerson (Vetsuisse Faculty, University of Zurich) for valuable assistance with the statistical analysis of the probability of detection.

335

336

337 **5 References**

338

339 Aguirre, J.S., Monis, A., Garcia de Fernando, G.D., 2014. Improvement in the lag phase
340 estimation of individual cells that have survived mild heat treatment. Int. J. Food Sci. Tec. 49,
341 884-894.

342

343 D`Arrigo, M., Garcia de Fernando, G.D., Velasco de Diego, R., Ordonez, J.A., George, S.M.,
344 Pin, C., 2006. Indirect measurement of the lag time distribution of single cells of *Listeria*
345 *innocua* in food. Appl. Environ. Microbiol.72, 253-258.

346

347 Baranyi, J., Pin, C., 1999. Estimating bacterial growth parameters by means of detection
348 times. Appl. Environ. Microbiol. 65, 732-736.

349

350 Breeuwer, P., Lardeau, A., Peterz, M., Joosten, H., 2003. Desiccation and heat tolerance of
351 *Enterobacter sakazakii.* J. Appl. Microbiol. 95, 967-973.

352

353 Caubilla-Barron, J., Iversen, C., Forsythe, SJ., 2004. The desiccation survival of *Enterobacter*
354 *sakazakii* and related Enterobacteriaceae. Abstr. 104th General meeting of American Society
355 for Microbiology, Washington D.C.

356

357 Dancer, G.I., Mah, J.H., Rhee, M.S., Hwang, I.G., Kang, D., 2009. Resistance of
358 *Enterobacter sakazakii* (*Cronobacter* spp.) to environmental stress. J. Appl. Microbiol. 107,
359 1606-1614.

360

361 Dukan, S., Nystrom, T., 1998. Bacterial senescence: stasis results in increased and differential
362 oxidation of cytoplasmic proteins leading to developmental induction of the heat shock
363 regulon. Gene. Dev.12, 3431-3441.
364
365 Dupont, C., Augustin, J.C., 2009. Influence of stress on single cell lag time and growth
366 probability for *Listeria monocytogenes* in Half Fraser broth. Appl. Environ. Microbiol. 75,
367 3069-3076.
368
369 Elfwing, A., LeMarc, Y., Baranyi, J., Ballagi, A., 2004. Observing growth and division of
370 large numbers of individual bacteria by image analysis. Appl. Environ. Microbiol. 70, 675-
371 678.
372
373 Francois, K., Devlieghere, F., Standaert, A.R., Geeraerd, A.H., van Impe, J.F., Debevere, J.,
374 2003. Modelling the individual cell lag phase: Isolating single cell: protocol development.
375 Lett. Appl. Microbiol. 37, 26-30.
376
377 Gnanou Besse, N., Dubois Brisonnet, F., Lafarge, V., Leclerc, V., 2000. Effect of various
378 environmental parameters on the recovery of sublethally salt-damaged and acid-damaged
379 *Listeria monocytogenes*. J. Appl. Microbiol. 89, 944-950.
380
381 Guillier, L., Augustin, J.C., 2006. Modelling the individual cell lag time distributions of
382 *Listeria monocytogenes* as a function of the physiological state and the growth conditions. Int.
383 J. Food Microbiol.111, 241-251.
384
385 Guillier, L., Pardon, P. Augustin, J.C., 2005. Influence of stress on individual lag time
386 distributions of *Listeria monocytogenes*. Appl. Environ. Microbiol. 71, 2940-2948.

Gurtler J.B., Beuchat, L.R., 2005. Performance of media for recovering stressed cells of *Enterobacter sakazakii* determined using spiral plating and ecometric techniques. Appl. Environ. Microbiol. 71, 7661-7669.

Iversen, C., Druggan, P., Schumacher, S., Lehner, A., Feer, C., Gschwend, K., Joosten, H., Stephan, R., 2008. Development of a novel screening method for the isolation of "Cronobacter" spp. (*Enterobacter sakazakii*). Appl. Environ. Microbiol. 74, 2550-2553.

Jongenburger, I., Reij, M.W., Boer, E.P.J., Gorris, L.G.M., Zwietering, M.H., 2011. Actual distribution of *Cronobacter* spp. in industrial batches of powdered infant formula and consequences for performance of sampling strategies. Int. J. Food Microbiol. 151, 62-69.

Kandhai, M.C., Reij, M.W., Grognou, C., van Schothorst, M., Gorris, L.G.M., Zwietering, M.H., 2006. Effects of preculturing conditions on lag time and specific growth rate of *Enterobacter sakzakii* in reconstituted powdered infant formula. Appl. Environ. Microbiol. 72, 2721-2729.

Li, Y., Odumeru, J.A., Griffiths, M., McKellar, R.C., 2006. Effect of environmental stresses on the mean and distribution of individual cell lag times of *Escherichia coli* O157:H7. Int. J. Food Microbiol. 110, 278-285.

Margot, H., Zwietering, M.H., Joosten, H., O`Mahony, E., Stephan, R., 2015. Evaluation of different buffered peptone water (BPW) based enrichment broths for detection of Gram-negative foodborne pathogens from various food matrices. Int. J. Food Microbiol. 214, 109-115.

Metris, A., George, S.M., Peck, M.W., Baranyi, J., 2003. Distribution of turbidity detection times produced by single cell-generated bacterial populations. J. Microbiol. Meth. 55, 821-827.

Miled Bennour, R., Ells, T.C., Pagotto, F.J., Farber, J.M., Kerouanton, A., Meheut, T., Colin, P., Joosten, H., Leclercq, A., Gnanou Besse, N., 2010. Genotypic and phenotypic characterisation of a collection of *Cronobacter* (*Enterobacter sakazakii*) isolates. Int. J. Food Microbiol. 139, 116-125.

Miled Bennour, R., Guillier, L., Neves, S., Augustin, J.C., Colin, P., Gnanou Besse, N., 2011. Individual cell lag time distributions of *Cronobacter* (*Enterobacter sakazakii)* and impact of pooling samples on its detection in powdered infant formula. Food Microbiol. 28, 648-655.

Nazarowec-White, M., Farber, J.M., 1997. Thermal resistance of *Enterobacter sakazakii* in reconstituted dried-infant formula. Lett. Appl. Microbiol. 24, 9-13.

Pin, C., Baranyi, J., 2008. Single-cell and population lag times as a function of cell age. Appl. Environ. Microb.74, 2534-2536.

Rolfe, M.D., Rice, C.J., Lucchini, S., Pin, C., Thompson, A., Cameron, A.D.S., Alston, M., Stringer, M.F., Betts, R.P., Baranyi, J., Peck, M.W., Hinton, J.C.D., 2012. Lag phase is a distinct growth phase that prepares bacteria for exponential growth and involves transient metal accumulation. J. Bacteriol. 194, 686-701.

Smelt, J.P.P.M., Otten, G.D., Bos, A.P., 2002. Modelling the effect of sublethaly injury on the distribution of the lag times of individual cells of *Lactobacillus plantarum*. Int. J. Food Microbiol. 73, 207-212.

Stephens, P.J., Joynson, J.A., Davies, K.W., Holbrook, R., Lappin-Scott, H.M., Humphrey, T.J., 1997. The use of an automated growth analyser to measure recovery times of single heat-injured *Salmonella* cells. J. Appl. Microbiol. 83, 449-455.

Swinnen, L.A.M., Bernearts, K., Dens, E.J.J., Geeraerd, A.H., Van Impe, J.F., 2004. Predictive modelling of the microbial lag phase: a review. Int. J. Food Microbiol. 94, 137-159.

Xu, Y.Z., Metris, A., Stasinopoulos, D.M., Forsythe, S.J., Sutherland, J.P., 2015. Effect of heat shock and recovery temperature on variability of single cell lag time of *Cronobacter turicensis*. Food Microbiol. 45, 195-204.